Alcohol oxidase

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Alcohol oxidase: A complex peroxisomal, oligomeric flavoprotein

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Abstract

Alcohol oxidase (AO) is the key enzyme of methanol metabolism in methylotrophic yeast species. It catalyses the first step of methanol catabolism, namely its oxidation to formaldehyde with concomitant production of hydrogen peroxide. In its mature active form, AO is a molecule of high molecular mass (600 kDa) that consists of eight identical subunits, each of which carry one non-covalently bound flavin adenine nucleotide (FAD) molecule as the prosthetic group. In vivo, the protein is compartmentalized into special cell organelles, termed peroxisomes.

AO is an abundant protein and its synthesis is strictly regulated by repression/derepression and induction mechanisms that occur at the transcriptional level. Various aspects of its sorting and assembly/activation render AO a unique protein.

Recent developments of AO synthesis, sorting and assembly/activation are highlighted in this paper.

Keywords: Alcohol oxidase; Protein sorting; Dofactor binding; Peroxisome

1. Introduction

Several yeast species, belonging to the genera *Pichia* and *Candida*, are able to utilize methanol as sole source of carbon and energy. Growth of these organisms on methanol requires the function of specific organelles, called peroxisomes, that contain the key enzymes of methanol metabolism. The first enzyme of methanol utilization is alcohol oxidase (AO; EC 1.1.3.13) that belongs to the family of glucose-methanol-choline (GMC) oxidoreductases [1]. The generalized name AO is adopted from the fact that in vitro the enzyme also oxidizes other short aliphatic alcohols [2]. AO protein and the regulation of the gene encoding AO (*AOX*) have been studied for decades for several reasons. Firstly, the promoter that controls *AOX* expression is known to be one of the strongest promoters in nature and, at the same time, one of most tightly controlled yeast promoters. These features render methylotrophic yeast species highly attractive hosts for heterologous gene expression [3–6]. Secondly, AO protein itself is of high industrial interest. It constitutes, for instance, the main component of an alcohol sensor [7–9] because of various favourable properties such as: (i) a high affinity for primary alcohols (μM range); (ii) the high stability of its active form; and (iii) its easy availability, as AO protein may comprise up to 30% of total cellular protein [10–12].

2. The *AOX* gene

Several genes encoding AO protein have been cloned to date from various organisms (Table 1), in particular

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Table 1
Genes encoding AO protein

Organism	Gene designation	Remarks	References
Methylotrophic yeast			
Hansenula polymorpha (*Pichia angusta*)	*MOX*		[85]
Pichia pastoris	*AOX1, AOX2*		[24]
Pichia methanolica	*AUG1 (MOD1), AUG2 (MOD2)*		[23,86]
Pichia pinus	*AOX1*		Genbank, GI:37694459; unpublished
Candida boidinii	*AOD1*		[87]
Filamentous fungi			
Penicillium chrysogenum	*AOX*, induced under conditions of penicillin production		[88]
Cladosporium fulvum	*AOX1*, induced by carbon starvation and during later stages of infection; deletion results in reduced pathogenicity		[89]
Helminthosporium (*Cochliobolus*) *victoriae*	–	Hv-p68; RNA-binding protein	[90]
Aspergillus nidulans	–	Hypothetical protein with high homology to AO from methylotrophic yeast	Genbank, GI:40747510; unpublished

from yeasts and filamentous fungi. Although AO proteins from filamentous fungi show a high degree of homology (65–70% identity) to those of methylotrophic yeast species, their function is not related to methanol metabolism as in yeasts. Various acronyms are used to indicate genes encoding AO (Table 1). For simplicity all genes identified thus far are designated *AOX* throughout this review.

3. *AOX* gene expression

The *Hansenula polymorpha AOX* gene [13–15] contains a large promoter region of approximately 1.5 kb that is tightly regulated. Three regulatory sequences have been identified within the *AOX* promoter, namely two upstream activation sequences, UAS1 and UAS2, as well as one upstream repressing sequence, URS1. These sequences were observed to bind regulatory proteins, named MBF1, MBF2 and MBF3 (*MOX* Binding Factor), respectively. However, these proteins have not been analysed further [13].

Expression of *AOX* is subject to strong carbon catabolite repression [16,17]. Under such conditions, the *AOX* promoter sequence (P_{AOX}) is organized in nucleosome structures [14], thus being unavailable for the transcription machinery. Prior to initiation of AO synthesis at derepressing or inducing conditions (for instance in the presence of glycerol or methanol), the gene needs to be liberated from nucleosomes via the function of the chromatin remodelling complex. Recently, we showed that HpSwi1p and HpSnf2p, proteins that are homologous to subunits of the SWI/SNF chromatin remodelling complex from *Saccharomyces cerevisiae*, may be involved in this process [18]. The ultimate level of *AOX* expression strongly depends on the cultivation conditions [16]. Also, differences exist in the regulation of *AOX* expression in different yeast species. In *H. polymorpha*, which has one *AOX* gene, low levels of AO can be observed at glucose-limiting conditions (e.g., glucose-containing batch cultures at the early stationary growth phase or in carbon-limited chemostats). The AO protein levels are enhanced when cells are grown in the presence of dihydroxyacetone or glycerol [16]. By contrast, in *Pichia pastoris* cells grown in presence of glycerol, no mRNA of *AOX* has been detected [19]. For very high AO protein levels, expression of the gene requires induction by methanol and can then increase to up to 30% of total cellular protein [11,12]. At these conditions, cells are loaded with peroxisomes (Fig. 1). Similar high AO levels are observed in glucose-limited chemostat cultures supplemented with choline as sole nitrogen source [20]. Interestingly, maximal induction of AO expression is also obtained in carbon-limited mixed substrate chemostat cultures supplemented with glucose and methanol. Both carbon sources are then completely utilized leading to increased biomass production, compared to single carbon source chemostat cultures [21].

In *P. pastoris* and *P. methanolica*, which contain two genes coding for AO (Table 1), differential expression of both genes is observed, a phenomenon that is strictly dependent on cultivation conditions [19,22,23]. This differential expression of both genes is related to the presence of different regulatory sequences [24].

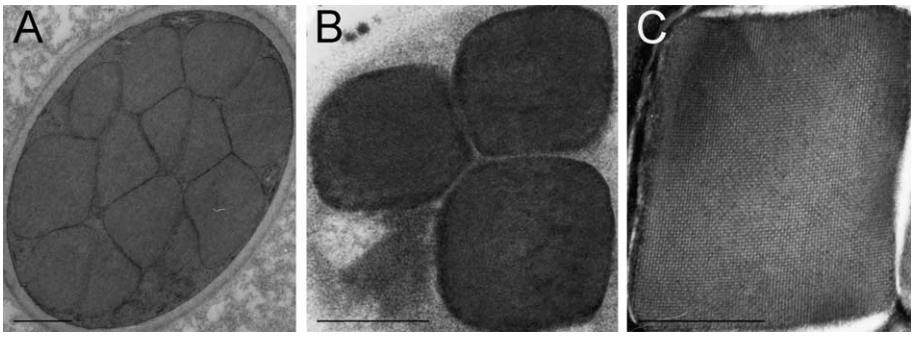

Fig. 1. Ultrathin sections of *H. polymorpha* cells grown in a methanol-limited chemostat ($D = 0.1\ h^{-1}$). (A) Presents an overview to demonstrate the abundance of peroxisomes in these cells. (B) AO activity staining after incubation of cells with methanol and $CeCl_3$. The enzyme activity is restricted to the peroxisomal matrix. (C) Shows a detail of a cryosection, stained with uranyl acetate, through a cell to demonstrate the completely crystalline matrix of the peroxisomes present in these cells. Most probably, the cuboid shape of the organelles is related to the presence of the crystalloid content. The bar represents 0.5 μm.

Recently, components involved in the regulation of *AOX* catabolite repression have been identified. These include a hexose transporter [25], a glucokinase and a hexokinase [26–28]. In the corresponding mutant strains, AO protein is produced at repressing cultivation conditions, e.g. during growth of cells on glucose. Comparably, *P. methanolica* and *H. polymorpha* cells mutated in acetyl CoA synthetase show the activity of AO in the presence of ethanol [29,30].

Several other mutants have been reported that produce AO in the presence of glucose [30–36], however the corresponding genes have not been identified.

4. Regulation of AO activity

4.1. Presence of modified FAD

Fine tuning of specific AO enzyme activity may be regulated via modifications of the co-factor FAD. One of these mechanisms involves the autoconversion of the naturally occurring FAD moiety to the modified form (mFAD or a-FAD) that was observed to specifically occur in AO [37]. mFAD was shown to represent a stereochemical FAD analogue, in which the C2 carbon of the ribityl chain has changed from the R to the S position [38,39]. The modification of FAD slightly decreases the V_{max} of AO but significantly decreases its K_m for methanol. Both effects are of significant physiological relevance in cultures that contain low amounts of methanol. Indeed, the content of mFAD in purified AO varies from 5% to 95% depending on the growth conditions of the cells and increasing at the stationary phase and at low concentrations of methanol [37,40]. In *P. methanolica*, mFAD was observed mainly in one isoform of AO (Mod1p) [40], a phenomenon which might largely contribute to the observed tenfold higher affinity of this AO isoform for methanol relative to the other isoform (Mod2p) [41]. Interestingly, AO was also shown to contain oxygen-stable flavin semiquinone moiety in amounts varying from 4 to 5 molecules per AO octamer, depending on the yeast species, that is not involved in enzyme catalysis [42]. The role of this flavin species was not elucidated.

4.2. Carbon catabolite inactivation

When methylotrophic yeast cells, grown on methanol, are shifted to conditions in which AO induction is repressed (i.e., on glucose or ethanol [43,44], the peroxisomes that harbour AO are subject to rapid proteolytic degradation. The mechanisms of the initiation of this process are not known. However, van der Klei et al. [45] have shown that tagging of peroxisomes for degradation is directed against components of the peroxisomal membrane and not to its matrix components. Besides degradative inactivation, modification inactivation of AO activity also is observed, e.g., upon exposure of methanol-induced peroxisome-deficient (*pex*) mutants to excess ethanol conditions [45]. This, however, as suggested by Murray and Duff [46], may be related to the formation of acetaldehyde from ethanol that inhibits AO activity. Comparable AO inactivation events occur during the peroxisome fission process [47] or when methanol-grown cells are exposed to methanol-excess conditions [45]. This latter AO inactivation effect may, however, be related to the high quantities of mFAD in AO prior to the shift, which has very high affinity for the methanol substrate.

4.3. Degradative AO inactivation

The degradative inactivation of AO upon a shift of the cells to glucose or ethanol – a process termed macropexophagy – is beyond the scope of this paper and it is reviewed elsewhere [48,49].

5. AO structure

As indicated before, AO belongs to the family of glucose-methanol-choline (GMC) oxidoreductases. In 1998, Kiess et al. [1] have aligned eleven proteins belonging to this family, including AO from *C. boidini* and *H. polymorpha*. Three major domains have been identified, namely an FAD-binding domain, a flavin attachment loop and a substrate-binding domain, as well as two minor domains, namely an FAD-covering loop and an extended FAD-binding domain. Next to these, AO and cholesterol oxidase contain specific inserted regions. In case of AO, two inserts are identified in the substrate-binding domain that are probably involved in AO-octamer formation [1]. The FAD-binding domain is the most conserved region and comprises four sequence regions distributed over the whole primary sequence. The first region is a common ADP-binding motif (βαβ) present in most FAD-binding proteins containing the characteristic nucleotide-binding site GXGXXG (amino acids 13–18 of AO, namely GGGSTG).

Despite numerous attempts to resolve the structure of AO, no three-dimensional model is available to date [50–52]. However, Botevá et al. [53] created a model of AO (Fig. 2) that is based on the known structure of glucose oxidase (GOX) from *Aspergillus niger* [54] that shares the highest homology to *H. polymorpha* AO (25% identity) among the known structures of the GMC oxidoreductases family. The structure suggests that the FAD molecule is buried in the protein matrix. This is in agreement with the finding that the FAD fluorescence of AO is twofold less intensive than that of FAD in solution [53]. Although GOX and AO share a strong conservation in the FAD-binding fold, KCN-induced release of FAD from the proteins in vitro had different effects. In case of GOX, release of FAD did not change the conformation of the protein and its enzyme activity was fully recovered upon addition of excess FAD, suggesting that FAD had re-associated to the protein. By contrast, the conformation of AO had drastically changed following FAD release and the dissociation of FAD from AO appeared to be irreversible [55].

In vivo, AO forms crystalloids in the peroxisomal matrix (Fig. 1C). Based on electron-microscopic analysis, the structural organization of AO crystalloids in vivo and of 2D crystalloids grown in vitro have been resolved [56,57]. The models predict an open structure of the AO crystal that allows diffusion throughout the intercrystal space of other peroxisomal proteins, e.g., dihydroxyacetone synthase (DHAS). However, DHAS is not a structural part of the crystalloid. The properties of another abundant peroxisomal enzyme, catalase, prevent this protein to diffuse into the crystalloid. Instead, catalase is located at the periphery of the organelle, at the edges of the crystalloid [58]. Hence, catalase is also not a structural part of the AO crystal as was suggested before by Osumi et al. [59]. The model of Vonck and van Bruggen [57] proposes that the unit cell of 2D AO crystalloids is composed of six AO octamers, organized in three orientations, and two large holes to accommodate other proteins. The formation of crystalloids in peroxisomes is believed to occur spontaneously, facilitated by high focal AO concentrations. In *H. polymorpha* mutants defective in matrix protein import, cytosolic AO crystalloids are formed [60,61].

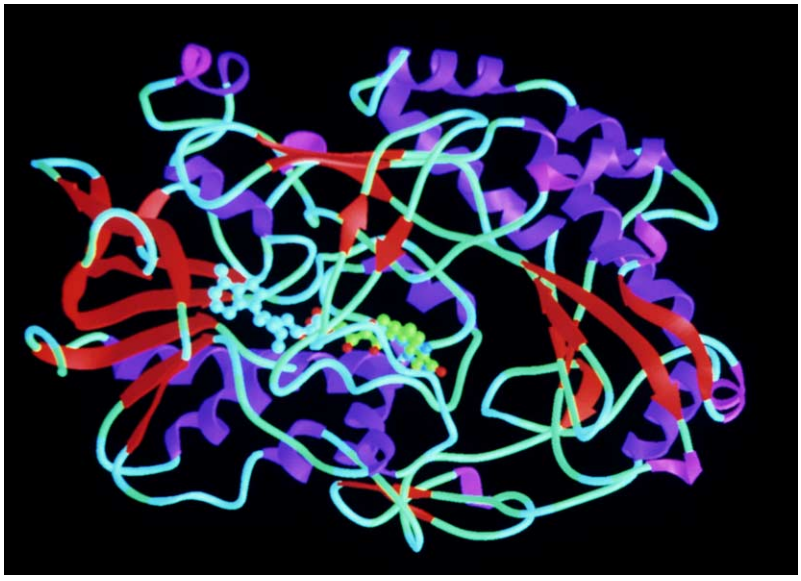

Fig. 2. Ribbon representation of the 3D model of *H. polymorpha* AO monomer (see [53]). α-helices are presented in purple, β-strands- in red. The ball-and stick model represents FAD.

6. The biosynthetic pathway of *H. polymorpha* AO

The process of AO import and assembly is not dependent on methanol-inducing conditions, because import and activation also occur in glucose-grown *H. polymorpha* cells in which the *AOX* gene is artificially expressed [62].

The details of the AO biosynthetic pathway are presented below.

6.1. FAD binding

Based on the analysis of a *H. polymorpha* riboflavin-deficient mutant (*rif1*) that was impaired in FAD biosynthesis, Evers et al. [63,64] have suggested FAD binding and subsequent AO maturation to take place in the peroxisomal lumen. At riboflavin-limiting conditions, *rif1* cells contained active peroxisomal AO in conjunction with a significant portion of the protein in the cytosol that lacked FAD [63,64]. These authors explained this partial impairment of AO import into peroxisomes as the result of the saturation of a putative peroxisomal FAD-binding factor. However, later data challenged this model and indicated that FAD binding occurs in the cytosol and is in fact essential to facilitate recognition of AO monomers by the PTS1 receptor Pex5p. The first direct proof for this assumption was obtained from the analysis of mutant AO protein that carried an amino acid substitution in the FAD-binding site (AO G15A) [65]. This mutant-AO indeed lacked FAD and was mislocalized to the cytosol, thereby supporting the idea that FAD binding in the cytosol was a prerequisite to allow AO import.

The first clues on the principles of AO assembly/activation came from studies of van Dijk et al. [66] who isolated a collection of *H. polymorpha* mutants defective in AO assembly. One strain from this collection, *ass3-110*, accumulated cytosolic, monomeric AO that lacked FAD and appeared to be mutated in the gene encoding pyruvate carboxylase (*PYC*). In yeast, Pyc protein is a cytosolic anapleurotic enzyme that replenishes the tricarboxylic acid cycle with oxaloacetate generated from pyruvate. Ozimek et al. [67] showed that HpPyc1p, but not its enzyme activity, is also essential for AO assembly, most likely as a helper protein in mediating FAD binding to AO precursor monomers. Interestingly, HpPyc1p was also capable to mediate AO activation in bakers' yeast strains in which the endogenous *PYC* genes were deleted (P. Ozimek et al., in preparation).

Mutational analysis of the *HpPYC1* gene resulted in the identification of a region in HpPyc1p that is specifically involved in its AO assembly function. This region shows relatively low homology to Pyc proteins from *S. cerevisiae* and is located in between the biotin carboxylation and transcarboxylation domains that are both involved in the enzyme activity of pyruvate carboxylases (P. Ozimek et al., in preparation).

6.2. AO protein import into peroxisomes

Sorting of AO to peroxisomes is dependent on the function of the PTS1 receptor, Pex5p [65,68,69]. Pex5p interacts with the Peroxisome Targeting Signal 1 (PTS1), a conserved tripeptide (consensus sequence – SKL) located at the extreme C-terminus of most peroxisomal matrix proteins [70,71]. Recognition and binding of the PTS1 motifs is mediated by a series of TPR domains (tetratricopeptide repeat), that are located in the C-terminal half of Pex5p [72]. However, Waterham et al. [69] showed that the PTS1 of *P. pastoris* AO was redundant for import. Similar results were obtained for *H. polymorpha* AO [65]. The latter authors proposed the existence of an alternative PTS that is being exposed upon initial AO folding due to the FAD binding event in the cytosol. Recognition of this novel PTS by HpPex5p is independent of its C-terminal half [65] but requires the function of the Pex5p N-terminus.

Several other proteins are known that require Pex5p for import into peroxisomes but lack a typical PTS1. These, among others, include acyl CoA oxidases from *Y. lipolytica* and *S. cerevisiae* [73,74] that are both flavo-enzymes. However, the mechanisms of their import show distinct differences. Using a two-hybrid approach, residues in ScPex5p have been identified that cluster in a small region that is involved in recognition of *S. cerevisiae* acyl CoA oxidase [74]. This region is, however, not conserved in either *Y. lipolytica* or *H. polymorpha* Pex5p. Acyl CoA oxidase from *Y. lipolytica* is a hetero-pentameric protein that is imported into peroxisomes in the hetero-oligomeric form. Two of the subunits, namely Aox2p and Aox3p, are essential for oligomerisation but not for FAD-binding to each subunit, which occurs prior to oligomerization. Apparently, the PTS of Ylacyl CoA oxidase is, similar to that of *H. polymorpha* AO,formed and exposed upon the formation of a tertiary/quaternary structure of the protein due to FAD binding. It is therefore tempting to speculate that these targeting signals are not stretches of amino acids (as in the PTS1 and PTS2) but comprise a more complex three-dimensional structure.

6.3. Assembly of AO

Our current knowledge on oligomeric protein import into peroxisomes suggests that most of the peroxisomal matrix proteins assemble in the cytosol to mature to enzymatically active proteins prior to import [75,76]. Most likely, AO is an exception to this rule. Indications for this came from work of Faber et al. [77] who used a temperature-sensitive mutant (*pex1-6*^{ts}) of *H. polymorpha*, which is impaired in peroxisome biogenesis at

43 °C but not at 37 °C. During growth of these cells at the restrictive temperature (43 °C), octameric AO accumulated in the cytosol. Upon a shift of these cells to the permissive temperature (37 °C), the cytosolic AO octamers were not imported in the newly formed peroxisomes. By contrast, dimeric DHAS was normally sorted to these new peroxisomes, formed at the permissive conditions. Also, in vitro binding experiments showed that octameric AO did not bind to HpPex5p, whereas dimeric DHAS normally associated with this receptor protein [77].

Details of the kinetics of AO and DHAS import and assembly in the methylotrophic yeast *C. boidinii* were monitored by Stewart et al. [78] via pulse-chase experiments. These studies confirmed that AO entered peroxisomes in its monomeric form. Prior to stable octamerisation an unstable intermediate form of oligomeric AO was detected. Whether such intermediates comprise a homo-oligomer of AO that contains less than eight subunits, or a complex of AO with a Pex5p or another peroxisomal protein remained unsolved.

A physiological rationale for the oligomerisation of AO in peroxisomes instead of in the cytosol came from the work of van der Klei et al. [79], showing that *H. polymorpha* mutants that contain enzymatically active AO in the cytosol are impaired in growth on methanol as a sole source of carbon and energy.

Obviously, the intriguing issue is how the cells prevent the cytosolic oligomerisation of AO. The most likely mechanism for this is the presence of cytosolic helper proteins that remain bound to AO from the moment it is capable of maturation, that is after FAD binding, until the monomers enter the peroxisomal lumen. The best candidate for such a factor would be Pex5p that, according to the extended shuttle model, enters the peroxisome together with the cargo (AO monomer) [80,81].

A schematic overview of the current knowledge of the AO biosynthetic pathway is presented in Fig. 3.

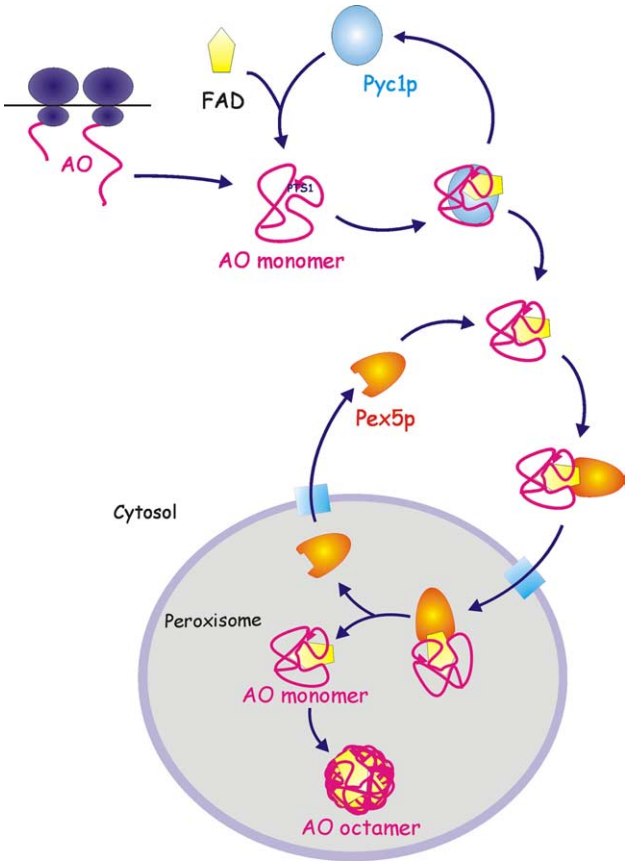

Fig. 3. A schematic overview of the current knowledge of the biosynthetic pathway of AO. AO synthesis takes place on free ribosomes. After synthesis, the FAD co-factor is bound to form the apomonomer. FAD binding requires the function of the cytosolic protein pyruvate carboxylase protein (Pyc1p). Subsequently, FAD-containing AO apomonomer is handed over to the PTS1 receptor protein Pex5p, which delivers the protein to the lumen of the target organelle. Upon entrance of the Pex5p–AO complex in the peroxisomal matrix, Pex5p is dissociated from the AO molecule, followed by assembly/activation of AO into the mature protein. After functioning, both Pyc1p and Pex5p are being recycled to allow another round of AO biosynthesis.

6.4. Peroxisomal proteases

Because of its complex biosynthetic pathway [65,67,78], which involves AO protein assembly/activation in the organellar lumen, the quality controls for AO assembly are expected to function in this lumen too. Indeed, Stewart et al. [78,82] identified a specific protease that was able to degrade in vitro synthesized AO monomers. This protein appeared to represent a component of the matrix of purified *C. boidinii* peroxisomes and was identified as a chymotrypsin-like protease of approximately 20 kDa, that formed oligomers of 80–90 kDa [82].

Recently, Kikuchi et al. [83] discovered a homolog of mitochondrial Lon protease in peroxisomes of rat liver. The substrate specificity of this protein was not studied but since other Lon proteases possess chaperone activity, the peroxisomal homolog was proposed to be involved in peroxisome biogenesis.

However, very recently Koek and van der Klei (unpublished results) isolated the *H. polymorpha* Lon homologue and showed that it can also degrade in vitro synthesized, monomeric AO proteins. These data are consistent with the view that in *H. polymorpha* peroxisomes and AO quality control mechanism may indeed exist.

7. Concluding remarks

Much effort has been put into the understanding of the regulation of the synthesis and complex assembly pathway of AO, ever since the protein was first

described [84]. Despite these efforts many aspects are still not clear. Resolving the 3D structure of AO would certainly benefit future developments of the field. However, considering the fact that AO undergoes conformational changes upon FAD-attachment and subsequent oligomerization, it may be important to study the early steps of AO assembly, e.g., in mutants that are defective in this process. Presumably, the changes of AO conformation are related to the well-documented alterations in susceptibility of the AO to proteolytic digestion (AO monomers are very sensitive, whereas octamers are resistant towards proteolytic digestion). Therefore, mild proteolytic treatment of cell extracts could be the method of choice to monitor the AO biosynthetic pathway.

Another interesting question that remains involves the details of the AO–Pex5p interaction. The intrinsic problem, however, is that mutations/truncations of Pex5p could interfere with the other functions of this protein (e.g., binding to proteins of the peroxisomal import machinery) and, on the other hand, that mutations in AO might readily interfere with FAD-binding. These two aspects render a mutant screen on this topic difficult. In this case, two-hybrid analysis seems to be the most favourable method to search for the sites of interactions between AO and Pex5p.

Also, the literature provides numerous data on the existence of mutants that are defective in either *AOX* expression regulation or AO assembly, but that have not yet been functionally complemented. This may lead to the identification of new genes that are involved in the biosynthesis of AO, what in turn would help to fill in the gaps in current knowledge of the field.

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References

[1] Kiess, M., Hecht, H.J. and Kalisz, H.M. (1998) Glucose oxidase from *Penicillium amagasakiense*. Primary structure and comparison with other glucose-methanol-choline (GMC) oxidoreductases. Eur. J. Biochem. 252, 90–99.

[2] Kato, N., Omori, Y., Tani, Y. and Ogata, K. (1976) Alcohol oxidases of *Kloeckera* sp. and *Hansenula polymorpha*. Catalytic properties and subunit structures. Eur. J. Biochem. 64, 341–350.

[3] Faber, K.N., Harder, W., Ab, G. and Veenhuis, M. (1995) Review: methylotrophic yeasts as factories for the production of foreign proteins. Yeast 11, 1331–1344.

[4] Rodriguez, L., Narciandi, R.E., Roca, H., Cremata, J., Montesinos, R., Rodriguez, E., Grillo, J.M., Muzio, V., Herrera, L.S. and Delgado, J.M. (1996) Invertase secretion in *Hansenula polymorpha* under the AOX1 promoter from *Pichia pastoris*. Yeast 12, 815–822.

[5] Raschke, W.C., Neiditch, B.R., Hendricks, M. and Cregg, J.M. (1996) Inducible expression of a heterologous protein in *Hansenula polymorpha* using the alcohol oxidase 1 promoter of *Pichia pastoris*. Gene 177, 163–167.

[6] Faber, K.N., Westra, S., Waterham, H.R., Keizer-Gunnink, I., Harder, W. and Veenhuis, M. (1996) Foreign gene expression in *Hansenula polymorpha*. A system for the synthesis of small functional peptides. Appl. Microbiol. Biotechnol. 45, 72–79.

[7] de Prada, A.G., Pena, N., Mena, M.L., Reviejo, A.J. and Pingarron, J.M. (2003) Graphite-teflon composite bienzyme amperometric biosensors for monitoring of alcohols. Biosens. Bioelectron. 18, 1279–1288.

[8] Hasunuma, T., Kuwabata, S., Fukusaki, E. and Kobayashi, A. (2004) Real-time quantification of methanol in plants using a hybrid alcohol oxidase-peroxidase biosensor. Anal. Chem. 76, 1500–1506.

[9] Mitsubayashi, K., Matsunaga, H., Nishio, G., Toda, S. and Nakanishi, Y. (2005) Bioelectronic sniffers for ethanol and acetaldehyde in breath air after drinking. Biosens. Bioelectron. 20, 1573–1579.

[10] van Dijken, J.P., Otto, R. and Harder, W. (1976) Growth of *Hansenula polymorpha* in a methanol-limited chemostat. Physiological responses due to the involvement of methanol oxidase as a key enzyme in methanol metabolism. Arch. Microbiol. 111, 137–144.

[11] Veenhuis, M., van Dijken, J.P. and Harder, W. (1983) The significance of peroxisomes in the metabolism of one-carbon compounds in yeasts. Adv. Microb. Physiol 24, 1–82.

[12] Guiseppin, M.L.F., van Eijk, H.M.L. and Bes, B.C.M. (1988) Molecular regulation of alcohol oxidase in *Hansenula polymorpha* in continuous cultures. Biotechnol. Bioeng. 32, 577–583.

[13] Godecke, S., Eckart, M., Janowicz, Z.A. and Hollenberg, C.P. (1994) Identification of sequences responsible for transcriptional regulation of the strongly expressed methanol oxidase-encoding gene in *Hansenula polymorpha*. Gene 139, 35–42.

[14] Costanzo, G., Di Mauro, E., Negri, R., Pereira, G. and Hollenberg, C. (1995) Multiple overlapping positions of nucleosomes with single in vivo rotational setting in the *Hansenula polymorpha* RNA polymerase II MOX promoter. J. Biol. Chem. 270, 11091–11097.

[15] Genu, V., Godecke, S., Hollenberg, C.P. and Pereira, G.G. (2003) The *Hansenula polymorpha MOX* gene presents two alternative transcription start points differentially utilized and sensitive to respiratory activity. Eur. J. Biochem. 270, 2467–2475.

[16] Roggenkamp, R., Janowicz, Z., Stanikowski, B. and Hollenberg, C.P. (1984) Biosynthesis and regulation of the peroxisomal methanol oxidase from the methylotrophic yeast *Hansenula polymorpha*. Mol. Gen. Genet. 194, 489–493.

[17] Sibirny, A.A., Titorenko, V., Efremov, B.D. and Tolstorukov, I.I. (1987) Multiplicity of mechanisms of carbon catabolite repression involved in the synthesis of alcohol oxidase in the methylotrophic yeast *Pichia pinus*. Yeast 3, 233–241.

[18] Ozimek, P., Lahtchev, K., Kiel, J.A., Veenhuis, M. and van der Klei, I.J. (2004) *Hansenula polymorpha* Swi1p and Snf2p are essential for methanol utilisation. FEMS Yeast Res. 4, 673–682.

[19] Cregg, J.M., Madden, K.R., Barringer, K.J., Thill, G.P. and Stillman, C.A. (1989) Functional characterization of the two alcohol oxidase genes from the yeast *Pichia pastoris*. Mol. Cell Biol. 9, 1316–1323.

[20] Zwart, K.B., Veenhuis, M. and Harder, W. (1983) Significance of yeast peroxisomes in the metabolism of choline and ethanolamine. Antonie Van Leeuwenhoek 49, 369–385.

[21] Egli, T., Kappeli, O. and Fiechter, A. (1982) Regulatory flexibility of methylotrophic yeast in chemostat cultures: simultaneous

assimilation of glucose and methanol at fixed dilution rate. Arch. Microbiol. 131, 1–7.
[22] Gruzman, M.B., Titorenko, V.I., Ashin, V.V., Lusta, K.A. and Trotsenko, Y.A. (1996) Multiple molecular forms of alcohol oxidase from the methylotrophic yeast *Pichia methanolica*. Biochemistry (Moscow) 61, 1537–1544.
[23] Nakagawa, T., Mukaiyama, H., Yurimoto, H., Sakai, Y. and Kato, N. (1999) Alcohol oxidase hybrid oligomers formed in vivo and in vitro. Yeast 15, 1223–1230.
[24] Koutz, P., Davis, G.R., Stillman, C., Barringer, K., Cregg, J. and Thill, G. (1989) Structural comparison of the *Pichia pastoris* alcohol oxidase genes. Yeast 5, 167–177.
[25] Stasyk, O.V., Stasyk, O.G., Komduur, J., Veenhuis, M., Cregg, J.M. and Sibirny, A.A. (2004) A hexose transporter homologue controls glucose repression in the methylotrophic yeast *Hansenula polymorpha*. J. Biol. Chem. 279, 8116–8125.
[26] Kramarenko, T., Karp, H., Jarviste, A. and Alamäe, T. (2000) Sugar repression in the methylotrophic yeast *Hansenula polymorpha* studied by using hexokinase-negative, glucokinase-negative and double kinase-negative mutants. Folia Microbiol. (Praha) 45, 521–529.
[27] Laht, S., Karp, H., Kotka, P., Jarviste, A. and Alamäe, T. (2002) Cloning and characterization of glucokinase from a methylotrophic yeast *Hansenula polymorpha*: different effects on glucose repression in *H. polymorpha* and *Saccharomyces cerevisiae*. Gene 296, 195–203.
[28] Karp, H., Jarviste, A., Kriegel, T.M. and Alamäe, T. (2003) Cloning and biochemical characterization of hexokinase from the methylotrophic yeast *Hansenula polymorpha*. Curr. Genet. 44, 268–276.
[29] Kulachkovsky, A.R., Moroz, O.M. and Sibirny, A.A. (1997) Impairment of peroxisome degradation in *Pichia methanolica* mutants defective in acetyl-CoA synthetase or isocitrate lyase. Yeast 13, 1043–1052.
[30] Moroz, O.M., Gonchar, M.V. and Sibirny, A.A. (2000) Efficient bioconversion of ethanol to acetaldehyde using a novel mutant strain of the methylotrophic yeast *Hansenula polymorpha*. Biotechnol. Bioeng. 68, 44–51.
[31] Tani, Y., Sawai, T. and Sakai, Y. (1988) Production of catalytic cells for formaldehyde production and alcohol oxidase by a catabolite repression-insensitive mutant of a methanol yeast *Candida boidinii* A5. Biotechnol. Bioeng. 32, 1165–1169.
[32] Roggenkamp, R. (1988) Constitutive appearance of peroxisomes in a regulatory mutant of the methylotrophic yeast *Hansenula polymorpha*. Mol. Gen. Genet. 213, 535–540.
[33] Alamäe, T. and Simisker, J. (1994) Isolation and preliminary characterization of *Pichia pinus* mutants insensitive to glucose repression. Yeast 10, 1459–1466.
[34] Alamäe, T. and Liiv, L. (1998) Glucose repression of maltase and methanol-oxidizing enzymes in the methylotrophic yeast *Hansenula polymorpha*: isolation and study of regulatory mutants. Folia Microbiol. (Praha) 43, 443–452.
[35] Parpinello, G., Berardi, E. and Strabbioli, R. (1998) A regulatory mutant of *Hansenula polymorpha* exhibiting methanol utilization metabolism and peroxisome proliferation in glucose. J. Bacteriol. 180, 2958–2967.
[36] Hristozova, T., Michailova, L., Tuneva, D., Gotcheva, V., Angelov, A. and Roshkova, Z. (2002) Mutant *Hansenula polymorpha* strain with constitutive alcohol oxidase and peroxisome biosynthesis. Z. Naturforsch. 57c, 858–862.
[37] Bystrykh, L.V., Dijkhuizen, L. and Harder, W. (1991) Modification of flavin adenine dinucleotide in alcohol oxidase of the yeast *Hansenula polymorpha*. J. Gen. Microbiol. 137, 2381–2386.
[38] Kellog, R.M., Kruizinga, W., Bystrykh, L.V., Dijkhuizen, L. and Harder, W. (1992) Structural analysis of a stereochemical modification of flavin adenine dinucleotide in alcohol oxidase from methylotrophic yeast. Tetrahedron 48, 4147–4162.
[39] van Berkel, W.J., Eppink, M.H. and Schreuder, H.A. (1994) Crystal structure of *p*-hydroxybenzoate hydroxylase reconstituted with the modified FAD present in alcohol oxidase from methylotrophic yeasts: evidence for an arabinoflavin. Protein Sci. 3, 2245–2253.
[40] Ashin, V.V. and Trotsenko, Y.A. (1998) Formation and distribution of modified FAD between isozymes of alcohol oxidase in the methylotrophic yeast *Pichia methanolica*. Biochemistry (Mosc.) 63, 1407–1413.
[41] Nakagawa, T., Mizumura, T., Mukaiyama, H., Miyaji, T., Yurimoto, H., Kato, N., Sakai, Y. and Tomizuka, N. (2002) Physiological role of the second alcohol oxidase gene MOD2 in the methylotrophic growth of *Pichia methanolica*. Yeast 19, 1067–1073.
[42] Mincey, T., Tayrien, G., Mildvan, A.S. and Abeles, R.H. (1980) Presence of a flavin semiquinone in methanol oxidase. Proc. Natl. Acad. Sci. USA 77, 7099–7101.
[43] Bruinenberg, P.G., Veenhuis, M., van Dijken, J.P., Duine, J.A. and Harder, W. (1982) A quantitative analysis of selective inactivation of peroxisomal enzymes in the yeast *Hansenula polymorpha* by high-performance liquid chromatography. FEMS Microbiol. Lett. 15, 45–50.
[44] Veenhuis, M., Douma, A.C., Harder, W. and Osumi, M. (1983) Degradation and turnover of peroxisomes in the yeast *Hansenula polymorpha* induced by selective inactivation of peroxisomal enzymes. Arch. Microbiol. 134, 193–203.
[45] van der Klei, I.J., Harder, W. and Veenhuis, M. (1991) Selective inactivation of alcohol oxidase in two peroxisome-deficient mutants of the yeast *Hansenula polymorpha*. Yeast 7, 813–821.
[46] Murray, W.D. and Duff, S.J. (1990) Catabolite inactivation in the methylotrophic yeast *Pichia pastoris*. Appl. Environ. Microbiol. 56, 2378–2383.
[47] Veenhuis, M., van Dijken, J.P. and Harder, W. (1976) Cytochemical studies on the localization of methanol oxidase and other oxidases in peroxisomes of methanol-grown *Hansenula polymorpha*. Arch. Microbiol. 111, 123–135.
[48] Leão, A.N. and Kiel, J.A. (2003) Peroxisome homeostasis in *Hansenula polymorpha*. FEMS Yeast Res. 4, 131–139.
[49] Bellu, A.R. and Kiel, J.A. (2003) Selective degradation of peroxisomes in yeasts. Microsc. Res. Tech. 61, 161–170.
[50] Boys, C.W., Hill, D.J., Stockley, P.G. and Woodward, J.R. (1989) Crystallization of alcohol oxidase from *Pichia pastoris*. J. Mol. Biol. 208, 211–212.
[51] van der Klei, I.J., Lawson, C.L., Rozeboom, H., Dijkstra, B.W., Veenhuis, M., Harder, W. and Hol, W.G. (1989) Use of electron microscopy in the examination of lattice defects in crystals of alcohol oxidase. FEBS Lett. 244, 213–216.
[52] Tykarska, E., Lebioda, L., Marchut, E., Steczko, J. and Stec, B. (1990) Crystallization of alcohol oxidase from *Pichia pastoris*. Secondary structure predictions indicate a domain with the eightfold beta/alpha-barrel fold. J. Protein Chem. 9, 83–86.
[53] Boteva, R., Visser, A.J., Filippi, B., Vriend, G., Veenhuis, M. and van der Klei, I.J. (1999) Conformational transitions accompanying oligomerization of yeast alcohol oxidase, a peroxisomal flavoenzyme. Biochemistry 38, 5034–5044.
[54] Hecht, H.J., Kalisz, H.M., Hendle, J., Schmid, R.D. and Schomburg, D. (1993) Crystal structure of glucose oxidase from *Aspergillus niger* refined at 2.3 Å resolution. J. Mol. Biol. 229, 153–172.
[55] Zlateva, T., Boteva, R., Filippi, B., Veenhuis, M. and van der Klei, I.J. (2001) Deflavination of flavo-oxidases by nucleophilic reagents. Biochim. Biophys. Acta 1548, 213–219.
[56] Veenhuis, M., Harder, W., van Dijken, J.P. and Mayer, F. (1981) Substructure of crystalline peroxisomes in methanol-grown *Hansenula polymorpha*: evidence for an in vivo crystal of alcohol oxidase. Mol. Cell Biol. 1, 949–957.

[57] Vonck, J. and van Bruggen, E.F. (1992) Architecture of peroxisomal alcohol oxidase crystals from the methylotrophic yeast *Hansenula polymorpha* as deduced by electron microscopy. J. Bacteriol. 174, 5391–5399.
[58] Keizer, I., Roggenkamp, R., Harder, W. and Veenhuis, M. (1992) Location of catalase in crystalline peroxisomes of methanol-grown *Hansenula polymorpha*. FEMS Microbiol. Lett. 72, 7–11.
[59] Osumi, M., Nagano, M., Yamada, N., Hosoi, J. and Yanagida, M. (1982) Three-dimensional structure of the crystalloid in the microbody of *Kloeckera* sp.: composite crystal model. J. Bacteriol. 151, 376–383.
[60] van der Klei, I.J., Sulter, G.J., Harder, W. and Veenhuis, M. (1991) Assembly of alcohol oxidase in the cytosol of a peroxisome-deficient mutant of *Hansenula polymorpha* – properties of the protein and architecture of the crystals. Yeast 7, 195–209.
[61] Waterham, H.R., Titorenko, V.I., Swaving, G.J., Harder, W. and Veenhuis, M. (1993) Peroxisomes in the methylotrophic yeast *Hansenula polymorpha* do not necessarily derive from pre-existing organelles. EMBO J. 12, 4785–4794.
[62] Distel, B., van der Ley, I., Veenhuis, M. and Tabak, H.F. (1988) Alcohol oxidase expressed under nonmethylotrophic conditions is imported, assembled, and enzymatically active in peroxisomes of *Hansenula polymorpha*. J. Cell Biol. 107, 1669–1675.
[63] Evers, M.E., Titorenko, V.I., van der Klei, I.J., Harder, W. and Veenhuis, M. (1994) Assembly of alcohol oxidase in peroxisomes of the yeast *Hansenula polymorpha* requires the cofactor flavin adenine dinucleotide. Mol. Biol. Cell 5, 829–837.
[64] Evers, M.E., Titorenko, V.I., Harder, W., van der Klei, I.J. and Veenhuis, M. (1996) Flavin adenine dinucleotide binding is the crucial step in alcohol oxidase assembly in the yeast *Hansenula polymorpha*. Yeast 12, 917–923.
[65] Gunkel, K., van Dijk, R., Veenhuis, M. and van der Klei, I.J. (2004) Routing of *Hansenula polymorpha* alcohol oxidase: an alternative peroxisomal protein-sorting machinery. Mol. Biol. Cell 15, 1347–1355.
[66] van Dijk, R., Lahtchev, K.L., Kram, A.M., van der Klei, I.J. and Veenhuis, M. (2002) Isolation of mutants of *Hansenula polymorpha* defective in the assembly of octameric alcohol oxidase. FEMS Yeast Res. 1, 257–263.
[67] Ozimek, P., van Dijk, R., Latchev, K., Gancedo, C., Wang, D.Y., van der Klei, I.J. and Veenhuis, M. (2003) Pyruvate carboxylase is an essential protein in the assembly of yeast peroxisomal oligomeric alcohol oxidase. Mol. Biol. Cell 14, 786–797.
[68] van der Klei, I.J., Hilbrands, R.E., Swaving, G.J., Waterham, H.R., Vrieling, E.G., Titorenko, V.I., Cregg, J.M., Harder, W. and Veenhuis, M. (1995) The *Hansenula polymorpha* PER3 gene is essential for the import of PTS1 proteins into the peroxisomal matrix. J. Biol. Chem. 270, 17229–17236.
[69] Waterham, H.R., Russell, K.A., Vries, Y. and Cregg, J.M. (1997) Peroxisomal targeting, import, and assembly of alcohol oxidase in *Pichia pastoris*. J. Cell Biol. 139, 1419–1431.
[70] Gould, S.J., Keller, G.A. and Subramani, S. (1987) Identification of a peroxisomal targeting signal at the carboxy terminus of firefly luciferase. J. Cell Biol. 105, 2923–2931.
[71] Swinkels, B.W., Gould, S.J. and Subramani, S. (1992) Targeting efficiencies of various permutations of the consensus C-terminal tripeptide peroxisomal targeting signal. FEBS Lett. 305, 133–136.
[72] Terlecky, S.R., Nuttley, W.M., McCollum, D., Sock, E. and Subramani, S. (1995) The *Pichia pastoris* peroxisomal protein PAS8p is the receptor for the C-terminal tripeptide peroxisomal targeting signal. EMBO J. 14, 3627–3634.
[73] Titorenko, V.I., Nicaud, J.M., Wang, H., Chan, H. and Rachubinski, R.A. (2002) Acyl-CoA oxidase is imported as a heteropentameric, cofactor-containing complex into peroxisomes of *Yarrowia lipolytica*. J. Cell Biol. 156, 481–494.
[74] Klein, A.T., van den Berg, M., Bottger, G., Tabak, H.F. and Distel, B. (2002) *Saccharomyces cerevisiae* acyl-CoA oxidase follows a novel, non-PTS1, import pathway into peroxisomes that is dependent on Pex5p. J. Biol. Chem. 277, 25011–25019.
[75] McNew, J.A. and Goodman, J.M. (1996) The targeting and assembly of peroxisomal proteins: some old rules do not apply. Trends Biochem. Sci. 21, 54–58.
[76] Gunkel, K., Veenhuis, M. and van der Klei, I.J. (in press). Protein translocation machineries: how organelles bring in matrix proteins. FEMS Yeast Res., doi:10.1016/j.femsyr.2005.03.004.
[77] Faber, K.N., van Dijk, R., Keizer-Gunnink, I., Koek, A., van der Klei, I.J. and Veenhuis, M. (2002) Import of assembled PTS1 proteins into peroxisomes of the yeast *Hansenula polymorpha*: yes and no. Biochim. Biophys. Acta 1591, 157–162.
[78] Stewart, M.Q., Esposito, R.D., Gowani, J. and Goodman, J.M. (2001) Alcohol oxidase and dihydroxyacetone synthase, the abundant peroxisomal proteins of methylotrophic yeasts, assemble in different cellular compartments. J. Cell Sci. 114, 2863–2868.
[79] van der Klei, I.J., Harder, W. and Veenhuis, M. (1991) Methanol metabolism in a peroxisome-deficient mutant of *Hansenula polymorpha*: a physiological study. Arch. Microbiol. 156, 15–23.
[80] van der Klei, I.J. and Veenhuis, M. (1996) Peroxisome biogenesis in the yeast *Hansenula polymorpha*: a structural and functional analysis. Ann. N.Y. Acad. Sci. 804, 47–59.
[81] Dammai, V. and Subramani, S. (2001) The human peroxisomal targeting signal receptor, Pex5p, is translocated into the peroxisomal matrix and recycled to the cytosol. Cell 105, 187–196.
[82] Stewart, M.Q., van Dijk, R., Veenhuis, M. and Goodman, J.M. (2002) Monomeric alcohol oxidase is preferentially digested by a novel protease from *Candida boidinii*. Biochim. Biophys. Acta 1542, 160–172.
[83] Kikuchi, M., Hatano, N., Yokota, S., Shimozawa, N., Imanaka, T. and Taniguchi, H. (2004) Proteomic analysis of rat liver peroxisome: presence of peroxisome-specific isozyme of Lon protease. J. Biol. Chem. 279, 421–428.
[84] Janssen, F.W., Kerwin, R.M. and Ruelius, H.W. (1965) Alcohol oxidase, a novel enzyme from a basidiomycete. Biochem. Biophys. Res. Commun. 20, 630–634.
[85] Ledeboer, A.M., Edens, L., Maat, J., Visser, C., Bos, J.W., Verrips, C.T., Janowicz, Z., Eckart, M., Roggenkamp, R. and Hollenberg, C.P. (1985) Molecular cloning and characterization of a gene coding for methanol oxidase in *Hansenula polymorpha*. Nucleic Acids Res. 13, 3063–3082.
[86] Raymond, C.K., Bukowski, T., Holderman, S.D., Ching, A.F., Vanaja, E. and Stamm, M.R. (1998) Development of the methylotrophic yeast *Pichia methanolica* for the expression of the 65 kilodalton isoform of human glutamate decarboxylase. Yeast 14, 11–23.
[87] Sakai, Y. and Tani, Y. (1992) Cloning and sequencing of the alcohol oxidase-encoding gene (AOD1) from the formaldehyde-producing asporogeneous methylotrophic yeast, *Candida boidinii* S2. Gene 114, 67–73.
[88] Holzmann, K., Schreiner, E. and Schwab, H. (2002) A *Penicillium chrysogenum* gene (AOX) identified by specific induction upon shifting pH encodes for a protein which shows high homology to fungal alcohol oxidases. Curr. Genet. 40, 339–344.
[89] Segers, G., Bradshaw, N., Archer, D., Blissett, K. and Oliver, R.P. (2001) Alcohol oxidase is a novel pathogenicity factor for *Cladosporium fulvum*, but aldehyde dehydrogenase is dispensable. Mol. Plant Microbe Interact. 14, 367–377.
[90] Soldevila, A.I. and Ghabrial, S.A. (2001) A novel alcohol oxidase/RNA-binding protein with affinity for mycovirus double-stranded RNA from the filamentous fungus *Helminthosporium* (*Cochliobolus*) *victoriae*: molecular and functional characterization. J. Biol. Chem. 276, 4652–4661.